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Introduction

Key to the kit is our proprietary DNA binding systems that allow the high efficient binding of DNA to our ezBind™ matrix while proteins and other impurities are removed by wash buffer. Nucleic acids are easily eluted with sterile water or Elution Buffer.

Unlike other kits in the markets, our patented plasmid purification kit has no chaotropic salts in the buffer. The purified DNA is guanidine/anion exchange resin residues free.

Plasmid isolated with traditional protocol normally contains high level of endotoxins (Lipopolysaccharides or LPS). For transfection of endotoxin sensitive cell lines or microinjection, the endotoxins should be removed before the applications. The EZgene™ endofree system uses a specially formulated buffer that extracts the endotoxin from the plasmid DNA. Two rounds of extraction will reduce the endotoxin level to 0.1 EU (Endotoxin) per μg of plasmid DNA. The endofree plasmid miniprep kit provides an efficient endotoxin removal step into the traditional purification procedure to produce transfection grade plasmid DNA.

This kit is designed for fast and efficient purification of plasmid DNA from 15 to 50 mL of *E. coli* culture. The midi column has a DNA binding capacity of 250 μg.

The purified endofree DNA is ready for downstream applications such as transfection of endotoxin-sensitive cell lines, primary cultured cells or microinjection.

Important Notes

Plasmid Copy Numbers: The yield of plasmid DNA depends on the origin of the replication and the size of the plasmid. The protocols are optimized for high copy number plasmid purification. For low copy number plasmids, both the culture volume and the buffer volume need to be scaled up 2 times. Please contact our customer service for further information and reference Table 1 for the commonly used plasmids,

Table 1 Commonly used plasmid.

Plasmid	Origin	Copy Numbers	Expected Yield (μg per 50 mL)
pSC101	pSC101	5	5
pACYC	P15A	10-12	5-10
pSuperCos	pMB1	10-20	10-20
pBR322	pMB1	15-20	10-20
pGEM^{R}	Muted pMB1	300-400	100-150
pBluescript^{R}	ColE1	300-500	100-200
pUC	Muted pMB1	500-700	150-250

Host Strains: The strains used for propagating plasmid have significant influence on yield. Host strains such as Top 10 and DH5 yield high-quality plasmid DNA. *endA*+ strains such as JM101, JM110, HB101, TG1 and their derivatives, normally have low plasmid yield due to either endogenous endonucleases or high carbohydrates released during lysis. We recommend transform plasmid to an *endA*- strain if the yield is not satisfactory. For purifying plasmid DNA from *endA*+ strains (Table 2), we recommend use product PD1712.

Table 2 *endA* strains of *E. Coli*.

EndA*- Strains of *E. Coli							
DH5α	DH1	DH21	JM106	JM109	SK2267	SRB	XLO
TOP10	DH10B	JM103	JM107	SK1590	MM294	Stbl2™	XL1-Blue
BJ5182	DH20	JM105	JM108	SK1592	Select96™	Stbl4™	XL10-Gold

EndA*+ Strains of *E. Coli							
C600	JM110	RR1	ABLE® C	CJ236	KW251	P2392	BL21(DE3)
HB101	TG1	TB1	ABLE® K	DH12S ™	LE392	PR700	BL21(DE3) pLysS
JM101	JM83	TKB1	HMS174	ES1301	M1061	Q358	BMH 71-18
All NM strains				All Y strains			

Optimal Cell Mass (OD_{600} x mL of Culture): This procedure is designed for isolating plasmid grown in standard LB medium (Luria Bertani) for 12 -16 hours to a density of OD_{600} 2.0 to 3.0. If rich mediums such as TB or 2xYT are used, make sure the cell density doesn't exceed 3.0 (OD_{600}). A high ratio of biomass over lysis buffers result in low DNA yield and purity. The midi column has an optimal biomass of 100-150. For example, if the OD_{600} is 3.0, the optimal culture volume should be 25-50 mL.

Culture Volume: Use a flask or tube 4 times bigger in volume than the culture medium to secure optimal condition for bacteria growth. Don't exceed the maximum culture volume suggested in the protocol. Incomplete lysis due to over amount of bacterial culture results in lower yield and less purity.

Storage and Stability

Buffer A1 should be stored at 4 ℃ once RNase A is added. All other materials can be stored at room temperature (22-25 ℃). The Guaranteed shelf life is 12 months from the date of purchase.

Before Starting

Alternative endotoxin removal procedures are provided. Protocol A removes endotoxin during the purification of plasmid DNA and Protocol B removes endotoxin after the purification of plasmid DNA.

Prepare all components and get all necessary materials ready by examining this instruction booklet and become familiar with each step.

Important Notes

- RNase A: It is stable for half a year under room temperature. Spin down the RNase A vial briefly. Add the RNase A solution to Buffer A1 and mix well before use. Store at 4 ℃.
- **Buffer B1 precipitates below room temperature. It is critical to warm up the buffer at 50 ℃ to dissolve the precipitates before use.**
- Keep the cap tightly closed for Buffer B1 after use.
- Make sure the availability of centrifuge, especially, after mixing the lysate with ethanol, the sample needs to be processed immediately by centrifugation.
- Centrifuge speed at 5,000 x g is recommended. In case the collection tube doesn't fit in high speed centrifuge rotor, use benchtop centrifuge and spin at 2,500 x g with double centrifugation time.
- ***Carry out all centrifugations at room temperature.***

Materials supplied by users

- 70% ethanol and 100% ethanol.
- High speed centrifuge and 30 mL high speed centrifuge tubes.
- 15 mL and 50 mL conical tubes.

Kit Contents

Catalog #	PD1415-00	PD1415-01	PD1415-02
Preps	2	10	25
EzBind™ Columns	2	10	25
Buffer A1	6 mL	30 mL	70 mL
Buffer B1	6 mL	30 mL	70 mL
Buffer N3	8 mL	40 mL	100 mL
Buffer KB	7 mL	35 mL	85 mL
EndoClean Buffer	2 mL	10 mL	25 mL
RNase A (20 mg/mL)	0.6 mg (30 μL)	3 mg (150 μL)	7 mg (350 μL)
Endofree Eluiton Buffer	3 mL	15 mL	50 mL
User Manual	1	1	1

Safety Information

- Buffer N3 contains acidic acid, wear gloves and protective eyewear when handling.
- Buffer N3 and KB contains chaotropic salts, which may form reactive compounds when combines with bleach. Do not add bleach or acidic solutions directly to the preparation waste.

EZgene™ EndoFree Plasmid Midiprep Spin Protocol

A. Removal of Endotoxin *during* Plasmid Purification

This protocol is designed for removing the endotoxin during the plasmid purification.

1. Inoculate **15-50 mL** LB containing appropriate antibiotic with 50 µL fresh starter culture. Incubate at 37 ℃ for 14-16 hours with vigorous shaking.

   Note: The best way to prepare a starter culture: Inoculate a single colony from a freshly grown selective plate into 1 mL LB medium containing the appropriate antibiotic and grow at 37 ℃ for 6-8 hours with vigorous shaking (~250 rpm).
   Note: Do not use a starter culture that has been stored at 4 ℃.
   Note: Do not grow starter culture directly from glycerol stock.
   Note: Do not use more than 50 mL culture or cell mass greater than 150.

2. Harvest the bacterial by centrifugation at 5,000 x g for 10 minutes at room temperature. Pour off the supernatant and blot the inverted tube on paper towels to remove residual medium.

3. Add **2.5 mL Buffer A1** (Add RNase A to **Buffer A1** before use) and completely resuspend bacterial pellet by vortexing or pipetting **(Complete resuspension is critical for optimal yields).**

4. Add **2.5 mL Buffer B1,** mix gently but thoroughly by inverting 5 times and incubate for 5 minutes to obtain a slightly clear lysate.

   Note: Do not incubate longer than 5 minutes. Over-incubating causes genomic DNA contamination and plasmid damage.

5. Add **600 µL Buffer N3**, mix immediately by inverting 5 times and sharp hand shaking for 5 times.

   Note: It is critical to mix the solution well. If the mixture still appears conglobated, brownish or viscous, more mix is required to completely neutralize the solution.

6. Transfer the lysate to a high-speed centrifuge tube and centrifuge at 13,000 x g for 10 minutes at room temperature.

   Note: Syringe filter (Supplied in PD1416 or purchase separately from Biomiga) could be used to filtrate the lysate if high-speed centrifuge is not available.

7. Transfer the clear lysate to a new high-speed centrifuge tube and add **0.1 volume** of **EndoClean Buffer**, vortex for 10s and incubate on ice for 10

minutes. Mix the sample several times without leaving ice.

Note: Use a serological pipet or a tip cut with a clean razor in the end to transfer the EndoClean Buffer.
Note: At room temperature (> 23℃), the sample becomes turbid after adding **EndoClean Buffer.** The solution becomes clear after incubating on ice.

8. Centrifuge at 13,000 x g for 10 min (Alternatively, the sample can be processed in a 15 mL conical tube and centrifuge at 2,500 x g for 15 min) at room temperature (the temperature must be greater than 23 ℃). The solution should separate into 2 phases, the upper clear phase contains DNA and the lower organic phase contains endotoxin. The two phases will not separate if the temperature is less than 23 ℃.

   Note: If phase partitioning is not observed after centrifugation：
   - Incubate the solution at 65℃ for 5 minutes. The solution becomes turbid again. And then repeat step 8.
   - Or add **200 µL Chloroform** (37℃), Mix well，repeat step 8.

   Note: Up to 99% of the endotoxin can be removed by extracting with the EndoClean buffer once. Another extraction is necessary if less than 0.1 EU (Endotoxin)/ µg of DNA is desired by repeating step 7-8.

9. Carefully transfer the clear supernatant into a 15 mL conical tube (avoid the interface precipitates). Add **3 mL Buffer N3** and **3 mL 100% ethanol.** Mix immediately by sharp hand shaking for 5 times. The mixture of ethanol/lysate needs to be transfer to the DNA column immediately.

10. Immediately transfer **6 mL** the **lysate/ethonal mix** into a DNA column with a 15 mL collection tube. Centrifuge at > 2,500 x g for 1 min at room temperature. Remove the column from the tube and discard the flow-through liquid. Reinsert the column to the collection tube.

11. Repeat step 10 till all the lysate/ethonal mix has been passed through the column.

12. **Optional:** Add **3.0 mL Buffer KB** into the spin column, centrifuge at > 2,500 x g for 1 minute. Remove the spin column from the tube and discard the flow-through. Put the column back to the collection tube.

    Note: Buffer KB is recommended for *endA*+ strains such as HB101, JM101, TG1 or their derived strains. It is not necessary for isolating DNA from *endA*- strains such as Top 10 and DH5a. Please reference Table 2 on page 3.

13. Add **5 mL 70% ethanol** into the column, centrifuge at > 2,500 x g for 1 min.

Remove the column from the tube and discard the flow through. Reinsert the column into the collection tube. Repeat step "13".

14. Centrifuge the column, **with the lid open**, at > 2,500 x g for 10 minutes. This step removes residual ethanol for optimal elution in next step.

    Note: Residual ethanol can be removed more efficiently with the column lid open. High centrifuge speed is suggested to remove the ethanol. It is critical to remove residual ethanol completely.

15. Carefully transfer the spin column into a clean 15 mL tube and add **0.5 mL Endofree Elution Buffer** to the center of the column and incubate for 1 minute at room temperature. Elute the DNA by centrifugation at > 2,500 x g for 5 minutes. If higher yield is desired, reload the eluate in the 15 mL tube to the column, incubate for 1 minute and centrifuge again.

    Note: Two elutions give rise to maximum DNA yield. Use less **Endofree Elution Buffer** if high concentration is desired.

    Note: The DNA is ready for downstream applications such as cloning/subcloning, RFLP, Library screening, *in vitro* translation, sequencing, transfection, and microinjection.

16. The DNA concentration can be calculated as follows,

    DNA concentration (μg/mL) = OD_{260nm} x 50 x dilution factor.

B. Removal of Endotoxin *after* Plasmid Purification

This protocol is designed for removing the endotoxin after the plasmid is purified.

1. Follow the protocol from Step 1 to 6 on page 6-7.

2. Transfer the lysate to a clean 15 mL conical tube and add **3 mL** of **Buffer N3** and **3 mL** of **100% ethanol**, mix well and go to step 10-15 on page 9-10.

3. After the plasmid is purified, add **0.1 volume** of **EndoClean Buffer** to the plasmid sample in a 2 mL centrifuge tube (For example, add **0.1 mL EndoClean Buffer** to **1 mL plasmid sample**). The solution becomes turbid after adding EndoClean Buffer.

4. Vortex the tube for 5s and put on ice for about 10 minutes Mix the sample several times without leaving ice. The solution becomes clean after incubating on ice.

5. Centrifuge at 12,000 x g at **room temperature** for 10 minutes **(the temperature must be greater than 23 ℃ for phase partitioning).**

   Note: If phase partitioning is not observed after centrifugation,
   - Incubate the solution at 65 ℃ for 5 minutes., and repeat step 5.
   - Or add **200 µL Chloroform** (37 ℃), votex for 10s, and repeat step 5.

6. Carefully transfer the upper clear layer solution to a 2 mL tube.

7. Precipitate plasmid DNA with **0.1 volume** of **3 M KAc (pH 5.2)** and **0.7 volume** of **Isopropanol**. Centrifuge at 12,000 x g for 10 minutes. Carefully decant.

8. Add **1 mL 70% ethanol** and centrifuge at 12,000 x g for 5 minutes. Carefully decant and air-dry the DNA for 30 minutes in a hood.

9. Resuspend the DNA with **Endofree Elution Buffer**.

   Note: The DNA is ready for downstream applications such as cloning/subcloning, RFLP, Library screening, *in vitro* translation, sequencing, transfection, and microinjection.

   DNA concentration (µg/mL) = $OD_{260\ nm}$ x 50 x dilution factor.

Purification of Low-Copy-Number Plasmid/Cosmid

The yield of low copy number plasmid is normally around 0.1–1 µg / mL of overnight culture. For isolating low copy number or medium copy number plasmid DNA, use the following guideline:

1. Culture volume: Use **2 x volumes** of the **high copy number** culture. Use up to 100 mL for midipreps.

2. Use **2 x volumes** of the **Buffer A1**, **Buffer B1**, **Buffer N3** and **100% ethanol**. Additional buffers can be purchased from Biomiga.

3. Use **same volumes** of **Wash Buffer (70% ethanol)** and **Endofree Elution Buffer.**

Trouble Shooting Guide

Problems	Possible Reasons	Suggested Improvements
Low Yield	Poor Cell lysis.	• Resuspend pellet thoroughly by votexing and pipetting prior adding Buffer B1. • Make fresh Buffer B1 if the cap had not been closed tightly. (Buffer B1: 0.2N NaOH and 1%SDS).
Low Yield	Bacterial culture overgrown or not fresh.	Grow bacterial 12-16 hours. Spin down cultures and store the pellet at -20 ℃. if the culture is not purified the same day. Do not store culture at 4 ℃ over night.
Low Yield	Low copy-number plasmid.	Increase culture volume and increase the volume of Buffer A1, B1, N3, and 100% ethanol according to instructions on page 9.
No DNA	Plasmid lost in Host *E.coli*	Prepare fresh culture.
Genomic DNA contamination	Over-time incubation after adding Buffer B1.	Do not vortex or mix aggressively after adding buffer B1. Do not incubate more than 5 minutes after adding Buffer B1.
RNA contamination	RNase A not added to Buffer A1.	Add RNase A to Buffer A1.
Plasmid DNA floats out of wells while running in agarose gel, DNA doesn't freeze or smell of ethanol	Ethanol traces not completely removed from column.	Make sure that no ethanol residual remaining in the silicon membrane before eluting the plasmid DNA. Re-centrifuge or vacuum again if necessary.

***FOR RESEARCH USE ONLY.**